Box 569 - A

DOCKET NO. : PUJ-0041

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Keith R. Marotti, Roger A. Poorman, Peter Andrew Wells, Dean L. Shinabarger

Serial No.: Not Yet Assigned **Group Art Unit:** Not Yet Assigned

Filing Date: Herewith **Examiner:** Not Yet Assigned

For: ELONGATION FACTOR P (EFP) AND ASSAYS AND ANTIMICROBIAL TREATMENTS RELATED TO THE SAME

EXPRESS MAIL LABEL NO: EL066387877US
DATE OF DEPOSIT: May 28, 1999

Box ☐ Patent Application

☒ Sequence ☐ Provisional ☐ Design

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☐ continuation-in-part of prior application number ___/_____.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☐ Provisional Application Cover Sheet.

☒ New or Revised Specification, including pages 1 to 46 containing:

- ☒ Specification
- ☒ Claims
- ☒ Abstract
- ☐ Substitute Specification, including Claims and Abstract.

☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the present application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application: ☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____ filed _____

_____

_____

_____.

☐ Signed Statement attached deleting inventor(s) named in the prior application.

☐ A Preliminary Amendment.

☐ _____ Sheets of ☐ Formal ☐ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____

_____.

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☐ The prior application is assigned of record to _____

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____.

☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☒ Diskette Containing DNA/Amino Acid Sequence Information.

☒ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application ________, is identical with that filed in Application Serial Number ________, filed ________. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: ______________________________.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: ______________________________
____
____.

FEE CALCULATION:

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			$75.00	$	$150.00	$
DESIGN APPLICATION			$155.00	$	$310.00	$
UTILITY APPLICATIONS BASE FEE			$380.00	$	$760.00	$760.00
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	394- 20 =	374	$9 each	$	$18 each	$6732.00
INDEP. CLAIMS	34- 3 =	31	$39 each	$	$78 each	$2418.00
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$130	$	$260	$260.00
ADDITIONAL FILING FEE				$		$0
TOTAL FILING FEE DUE				$		$10430.00

☒ A Check is enclosed in the amount of $ 10430.00 .

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

☐ The foregoing amount due.

☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.

☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).

☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-

identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: May 28, 1999

Paul K. Legaard
Registration No. 38,534

Woodcock Washburn Kurtz
Mackiewicz & Norris LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

ELONGATION FACTOR P (EFP) AND ASSAYS AND ANTIMICROBIAL TREATMENTS RELATED TO THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to provisional U.S. Application Serial No. 60/117,473 filed January 27, 1999, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention is directed, in part, to methods of using elongation factor p (efp) and related constituents of ribosomal complexes which comprise efp, the 50S ribosomal subunit, the 30S ribosomal subunit, the 70S ribosome, and related proteins, cofactors and enzymes to identify compounds that modulate prokaryotic cell function. Antibiotic compounds affecting such cell functions and methods of using those compounds to treat microbial infections in mammals are also described.

BACKGROUND OF THE INVENTION

An important catalytic function of ribosomes is the synthesis of peptide bonds. Various studies have suggested that the 70S ribosome, which is comprised of the 30S and 50S ribosomal subunits, is important for protein synthesis.

Models of protein synthesis assume that once the aminoacyl-tRNA is bound to the ribosomal A-site of the 70S ribosome complex, peptidyltransferase, an integral part of the 50S ribosomal subunit, can condense all twenty-one aminoacyl-tRNAs with equal efficiency, without intervention of exogenous proteins and GTP. However, several studies indicate that *in vitro*, the peptidyltransferase condenses predominantly hydrophobic amino acids. Peptide bond synthesis *in vitro* is also dependent upon aminoacyl moieties. In particular, prokaryotic 70S ribosomes cannot efficiently incorporate certain amino acids into polypeptides from cytidyl aminoacyl-adenosine (CA) (analogues of the 3'-terminal end of aminoacyl-tRNAs). As well, several antibiotics, such as anisomycin and chloramphenicol, inhibit peptide bond synthesis with some aminoacyl-tRNAs and not with others.

A prokaryotic gene encoding one of several known types of elongation factor proteins, elongation factor p (efp) was cloned and sequenced. Aoki *et al., Nucleic Acids Research*, 1991 (19), pp. 6215-6220, the disclosure of which is incorporated herein by reference in its entirety. Efp has been found to be essential for cell viability. Efp stimulates the efficiency of the peptidyltransferase activity of procaryotic ribosomes between fMet-$tRNA_f^{Met}$ and analogues of various aminoacyl-tRNAs. For example, the K' for the cytidyl(3'-5')-[2'(3')-O-L-CA-Gly is enhanced 50-fold, whereas that for CA-Phe is essentially unaltered by efp. Efp may modulate the efficiency of protein synthesis by controlling the rate of synthesis of certain peptide bonds. There are 800-900 molecules of efp per *E. coli*, or about 0.1 to 0.2 copy per ribosome, suggesting that efp may function catalytically in the cell. The preparation and isolation of efp can be found in M.C. Ganoza *et al., Eur. J. Biochem.*, 1985, vol. 146, pp. 287-294, and/or D-G. Chung et al. Chapter 4, pp. 69-80 of Ribosomes and Protein Synthesis, A Practical Approach, edited by G. Spedding, 1990, IRL Press at Oxford University Press, Oxford, NY. and Tokyo, the disclosures of which are incorporated herein by reference in their entirety.

The requirements of peptide-bond and ester-bond formation stimulated by efp have been studied with fMet-$tRNA_f^{Met}$ bound to 30S subunits and native or reconstituted 50S subunits. Efp functions in both peptide- and ester-bond synthesis promoted by the peptidyltransferase. The L16 protein (N-terminal fragment) of the 50S subunit is required for the efp-mediated synthesis of peptide bonds, whereas the L11, L15, and L7/L12 are not

required in this reaction, suggesting that efp may function at a different ribosomal site than most other translation factors.

Of interest is the fact that efp differentially stimulates peptide bond synthesis when various amino acids are covalently linked to aminoacyl-adenosine (CA). It is possible that efp preferentially acts on weak acceptors for the peptidyltransferase. The specific mechanism whereby efp stimulates bond synthesis is not entirely clear. Efp may help accommodate fMet-tRNA$_f^{Met}$ or peptidyl-tRNAs, or both, within the active center of the peptidyltransferase or it could affect peptidyltransferase directly.

The position occupied by each species of aminoacyl-tRNA on the ribosomes has been studied using antibiotics that are known to inhibit specific sites on the ribosome. Two types of A sites can be distinguished by their different reactivities towards specific antibiotics. The first site (of the i type) occur after fMet-tRNA$_f^{Met}$ has directly entered the ribosomal P-site, where the E-site is free. The second A-site (of the e type) is the one normally used to bind aminoacyl-tRNAs to 70S ribosomes during the course of chain elongation.

The antibiotics neomycin, thiostrepton, and hygromycin appear to inhibit translocation and occupation of the A site, but they inhibit only about 20% or have no effect on efp reaction. These antibiotics also have no effect on formation of the fMet-tRNA$_f^{Met}$/ribosome translation complex nor on the peptide-bond synthesis which occurs in the absence of efp.

Streptomycin at 2 x 10^{-5} M, which causes misreading and also inhibits A-site occupation of the e type, is a potent inhibitor of the efp-mediated reaction. The efp-mediated reaction is one in which purified efp is added to a translation complex of fmet-tRNA$_f^{Met}$:70S ribosome:mRNA; and then puromycin or an appropriate amino acid-charged tRNA is added. Efp mediates the formation of a peptide bond between the fmet and the second amino acid. Streptomycin is known to interact with two sites on the 16S rRNA of the 30S subunit. It is unknown, however, whether streptomycin acts to directly inhibit efp.

Lincomycin inhibits peptidyltransferase and occupation of the A-site of the e type. Lincomycin has marginal effects on the synthesis of polyphenylalanine, but inhibits the puromycin reaction and nullifies the ability of efp to stimulate synthesis of peptide bonds.

Erythromycin, also inhibits peptidyltransferase and it destabilizes the peptidyl-tRNA/ribosome/mRNA complex but it has no apparent effect on the efp reaction at 5×10^{-5}.

The present invention involves the surprising discovery of the critical role that efp may have in the procaryotic cell, and its role as a key component in the search for novel antimicrobial agents. These and other aspects of the invention are described below.

SUMMARY OF THE INVENTION

There is a need for more rapid and direct methods to screen compounds which may modulate ribosome mediated peptide bond formation. Such screening assays may discover new and useful antibiotics. New screens to detect and characterize compounds that affect efp and its functioning in the 70S ribosome, the 50S and 30S ribosomal subunits, and related proteins are disclosed herein. Newly discovered compounds or agents may promote cell death. The new understanding of the mechanism of action of known antimicrobials disclosed here may extend the usefulness of those antimicrobial agents.

Because of the surprising discovery disclosed here for the critical role that efp plays in the procaryotic cell, we can now disclose several aspects of this invention. Described herein are new methods or procedures to screen for, detect and/or characterize new compounds that modulate the function of efp in the prokaryotic cell. These methods or procedures include new *in vitro* methods as well as new *in vivo* methods.

In some embodiments of the invention, methods for identifying a compound which modulates activity of a prokaryotic elongation factor p in an *in vitro* assay, a cell based assay to determine the affect of the compound on cell function, a cell free extract assay to determine the affect of the compound on cell function are provided. The *in vitro* assay preferably comprises the steps of exposing elongation factor p with a compound, determining whether the compound modifies activity of the elongation factor p, and the cell-based assay preferably comprises determining whether the compound modifies activity of cell function. In some embodiments of the invention, the *in vitro* assay comprises determining whether the activity of the elongation factor p is decreased, determining whether the elongation factor p binds to the compound, determining whether the compound interferes with a function of the elongation factor p, or determining whether the compound interferes with a protein essential to the function of the elongation factor p such as the protein known as L16, or determining

whether the compound binds to the ribosome or some component thereof that prevents the binding of efp to the ribosome and therefore interferes with the proper functioning of efp. In some embodiments of the invention, the step comprises measuring association of the compound with elongation factor p. In some embodiments of the invention, disclosures are to methods for determining whether a compound decreases a function of the cell.

Also disclosed herein are new methods or procedures to screen for, detect and/or characterize new compounds that modulate the function of the 30S ribosomal subunit when it interacts with efp in the prokaryotic cell. These methods or procedures include new *in vitro* methods as well as new *in vivo* methods.

In some embodiments of the invention, disclosures are to methods for determining whether a compound modulates the function of the 30S ribosome, this can be accomplished in a variety of ways, including but not limited to determining whether the compound inhibits binding of fmet-tRNA or mRNA to the 70S ribosome; determining whether the compound prevents the 50S subunit from binding to the 30S subunit, thereby preventing formation of a functional 70S ribosome; determining whether the compound inhibits the binding of any aminoacyl-tRNA to the ribosome; and determining whether a compound prevents the binding of initiation factor 1, initiation factor 2, initiation factor 3, or other factors necessary for formation of the initiation of the initiation complex or first peptide bond synthesis. In some embodiments of the invention, under *in vitro* conditions, the third step comprises measuring the presence of initiation complex in the cell, wherein a decrease in the amount of the complex confirms that the compound interferes with the interaction of efp and the 30S ribosome. In some embodiments, the third step comprises measuring affinity or displacement of fmet-tRNA to the complex, wherein a low affinity indicates that the compound interacts with the 30S complex containing elongation factor p. Following these procedures the compounds can then be exposed to cell based assays to determine the viability of the cells treated with the compounds.

Also disclosed herein are new methods or procedures to screen for, detect and/or characterize new compounds that modulate the function of the 50S ribosomal subunit when it interacts with efp in the prokaryotic cell. These methods or procedures include new *in vitro* methods as well as new *in vivo* methods.

In some embodiments of the invention, disclosures are to methods for determining whether a compound modulates the function of the 50S ribosome, this can be accomplished in a variety of ways, including but not limited to determining whether the compound inhibits binding of fmet-tRNA or mRNA to the 70S ribosome; determining whether the compound inhibits formation of the first peptide bond between fmet and the second amino acid; determining whether the compound prevents the 50S subunit from binding to the 30S subunit, thereby preventing formation of a functional 70S ribosome; determining whether the compound inhibits the binding of any aminoacyl-tRNA to the ribosome; and determining whether a compound prevents the binding of initiation factor 1, initiation factor 2, initiation factor 3, or other factors necessary for formation of the initiation of the initiation complex or first peptide bond synthesis. In some embodiments of the invention, under *in vitro* conditions, the third step comprises measuring the presence of initiation complex in the cell, wherein a decrease in the amount of the complex confirms that the compound interferes with the interaction of efp and the 50S ribosome. In some embodiments of the invention, the third step comprises measuring affinity or displacement of fmet-tRNA to the complex, wherein a low affinity indicates that the compound interacts with the 50S complex containing elongation factor p. Following these procedures the compounds can then be exposed to cell-based assays to determine the viability of the cells treated with the compounds.

Also disclosed herein are new methods or procedures to screen for, detect and/or characterize new compounds that modulate the function of the 70S ribosome when it interacts with efp in the prokaryotic cell. These methods or procedures include new *in vitro* methods as well as new *in vivo* methods.

In some embodiments of the invention, disclosures are to methods for determining whether a compound modulates the function of the 70S ribosome, this can be accomplished in a variety of ways, including but not limited to including but not limited to determining whether the compound inhibits binding of fmet-tRNA or mRNA to the 70S ribosome; determining whether the compound inhibits formation of the first peptide bond between fmet and the second amino acid; determining whether the compound prevents the 50S subunit from binding to the 30S subunit, thereby preventing formation of a functional 70S ribosome; determining whether the compound inhibits the binding of any aminoacyl-tRNA to the ribosome; and determining whether a compound prevents the binding of initiation factor

1, initiation factor 2, initiation factor 3, or other factors necessary for formation of the initiation of the initiation complex or first peptide bond synthesis. In some embodiments of the invention, the third step comprises measuring the presence of initiation complex, wherein a decrease in the amount of the complex confirms that the compound interferes with the interaction of efp and the 70S ribosome. In some embodiments of the invention, the third step comprises measuring affinity or displacement of fmet-tRNA to the complex, wherein a low affinity indicates that the compound interacts with the 70S complex containing elongation factor p. Following these procedures the compounds can then be exposed to cell-based assays to determine the viability of the cells treated with the compounds.

In some embodiments of the invention a eukaryote protein known as eIF5A, a protein that performs a similar function in eukaryote cells as the efp protein does in prokaryotic cells is used to further identify and characterize compounds that have little or no modulating effect or inhibiting effect on eIF5A but that do modulate or inhibit efp. New methods or procedures, that employ all of the methods described above, to detect or characterize a new compound are then combined with the additional steps of determining that compounds modulating activity on the eukaryote version of efp (or eIF5A) and then comparing the modulating activity of the newly identified compound on prokaryotic efp with its modulating activity on eIF5A in order to identify or characterize compounds that inhibit efp activity on prokaryotic cells but have little adverse affect on the activity of eIF5A.

Also disclosed herein are new methods or procedures that allow the inhibition or control the growth of microbial organisms. Methods which disclose compounds that modulate the function of efp in the prokaryotic cell.

One important aspect of this invention is the discovery of the use of known compounds that can now be used to interfere with the important function of the efp protein.

The present invention further provides methods of modulating the activity of a bacterial elongation factor p comprising contacting the protein or a cell containing the protein with an oxazolidinone compound.

The present invention further provides methods of modulating the activity of a bacterial 30S ribosomal subunit comprising contacting the protein or a cell containing the subunit with an oxazolidinone.

The present invention further provides methods of modulating the activity of a bacterial 50S ribosomal subunit comprising contacting the protein or a cell containing the subunit with an oxazolidinone.

The present invention further provides methods of modulating the activity of a bacterial 70S ribosome comprising contacting the protein or a cell containing the subunit with an oxazolidinone.

The present invention further provides methods of modulating the activity of a bacterial L16 protein comprising contacting the protein or a cell containing the subunit with an oxazolidinone.

The present invention further provides methods of modulating the activity of a bacterial elongation factor p comprising contacting the protein or a cell containing the protein with an oxazolidinone type of compound.

These and other aspects of the invention are described in greater detail below.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Various definitions are made throughout this document. Most words have the meaning that would be attributed to those words by one skilled in the art. Words specifically defined either below or elsewhere in this document have the meaning provided in the context of invention as a whole and as are typically understood by those skilled in the art.

As used herein, the term "activity" refers to a variety of measurable indicia suggesting or revealing binding, either direct or indirect; affecting a response, *i.e.* having a measurable affect in response to some exposure or stimulus, including, for example, the affinity of the compound for directly binding efp or a ribosome, or, for example, measurement of amounts of upstream or downstream proteins or other similar functions after some stimulus or event.

As used herein, the term "affects" means either a decrease or increase in the amount or quality of a particular cell function in response to some stimulus, exposure or event.

As used herein, the term "binding" means the physical interaction between two proteins or compounds or associated proteins or compounds or combinations thereof. Binding includes ionic, non-ionic, Hydrogen bonds, Van der Waals, hydrophobic interactions, etc. The physical interaction, the binding, can be either direct or indirect, indirect being through or

because of another protein or compound. Direct binding refers to interactions that do not take place through or because of another protein or compound but instead are without other substantial chemical intermediates.

As used herein, the phrase "cell function" is defined to include all aspects of functionality of cells including cell viability but, especially, cell growth.

As used herein, the term "compound" means any identifiable chemical or molecule, small molecule, peptide, protein, sugar, natural or synthetic, or a discrete agent such as a specific amount of light, energy or temperature that is suspected to potentially interact with the process or system of interest, here typically efp, 30S, 50S, 70S ribosomes and related proteins.

As used herein, the term "contacting" means either direct or indirect, *in vitro* or *in vivo* administration of a compound to target, where the target may be a protein, ribosome, portion of a cellular system, whole cell, tissue, or mammal. The target may be in an *in vitro* or *in vivo* system with any number of buffers, salts, solutions etc.

As used herein, the phrases and terms "elongation factor p", "efp","ef-p", "EFP", or "EF-P" refers to the prokaryotic protein having a function like that of the efp protein isolated from *E. coli* or various other bacteria or recombinant versions of that protein such as that described by M.C. Ganoza *et al., Eur. J. Biochem* 1985, vol. 146, pp. 287-294, and H. Aoki *et al. Biochimie* (1997) vol. 79, pp. 7-11, Aoki *et al., Nucleic Acids Research*, 1991 (19), pp. 6215-6220, the disclosures of which are incorporated herein by reference in their entirety. The nucleic acid sequence of one or more of such proteins are provided in the references and the specification below. The EF-P differs from EF-Tu and EF-G in that it does not require GTP for its activity. Efp can be cloned, synthesized, or otherwise manipulated and if a version is made active according to any of the screens described here or in the references cited then that protein should be considered an efp protein.

As used herein, the term "effects" means either a decrease or increase in the amount or quality of a particular cell function in response to some stimulus, exposure or event.

As used herein, the phrase "first peptide bond reaction" means the joining of the I carboxyl group of formylmethionine to the I amino group of another amino acid.

As used herein, the phrase "formation of the initiation complex" means formation of a complex containing N formylmethionyl tRNA, 30S subunit, mRNA, GTP and the initiation factors IF1, IF2 and IF3.

As used herein, the term "interacting" means direct binding, including selective or specific binding, to a constituent of the ribosomal complex such that cell function is effected.

As used herein, the term "L16" means the L16 prokaryotic protein involved in bacterial protein synthesis as described in H. Aoki, *et al.*, *Molecular Characterization of the Procaryotic Efp Gene Product Involved in a Peptidyltransferase Reaction, Biochimie* (1997) vol. 79, pp. 7-11, the disclosure of which is incorporated herein by reference in its entirety.

As used herein, the terms "modulates" or "modifies" means an increase or decrease in the amount, quality, or effect of a particular activity or protein.

As used herein, the phrase "70S ribosome" means a prokaryotic ribonucleoprotein particle with a sedimentation coefficient of 70S that can be dissociated into a large subunit of 50S and a small subunit of 30S.

As used herein, the phrase "50S ribosome" or "50S subunit" means a prokaryotic ribonucleoprotein particle with a sedimentation coefficient of 50S that can be dissociated from a 70S ribosome.

As used herein, the phrase "30S ribosome" or "30S subunit" means a prokaryotic ribonucleoprotein particle with a sedimentation coefficient of 30S that can be dissociated from a 70S ribosome.

As used herein, the phrase "peptide bond donor" means any compound that has a free amino group capable of forming a peptide bond with an amino acid. Preferred peptide bond donors include, but are not limited to, puromycin or a puromycin analog, or any amino acyl-tRNA or an analog of amino acyl-tRNA.

As used herein, the term "oxazolidinone" means a compound of the class known as oxazolidinones, including the compounds described in U.S. Serial Numbers 07/438,759, 07/553,795, 08/006,596, 07/882,407, 07/786,107, 07/831,213, 08/233,903, 08/119,279, 08/226,158, 08/155,988, 08/329,717, 07/909,387, 08/339,979, 08/384,278, 08/875,800, 07/880,432, 08/610,031, 08/332,822, 07/988,589, 08/003,778, 08/066,356,

08/438,705, 60/015,499, 60/003,149, 09/138,205, 09/138,209, 08/696,313, 60/012,316, 08/803,469, 60/003,838, 08/709,998, 60/008,554, 08/762,478, 60/007,371, 08/850,424, 60/048,342, 09/080,751, 60/052,907, 60/064,746, 09/111,995, 60/064,738, 60/065,376, 60/067,830, 60/089,498, 60/100,185, 09/081,164, 60/088,283, 60/092,765, 07/244,988, 07/253,850; European Patents EP 0500686, EP 0610265, EP 0673370; PCT Application Numbers PCT/US90/06220, PCT/US94/08904, PCT/US94/10582, PCT/US95/02972, PCT/US95/10992, PCT/US93/04850, PCT/US95/12751, PCT/US96/00718, PCT/US93/03570, PCT/US93/09589, PCT/US96/05202, PCT/US97/03458, PCT/US96/12766, PCT/US97/01970, PCT/US96/14135, PCT/US96/19149, PCT/US96/17120, PCT/US98/09889, PCT/US98/13437;and U.S. Patent Numbers 5,700,799, 5,719,154, 5,547,950, 5,523,403, 5,668,286, 5,652,238, 5,688,792, 5,247,090, 5,231,188, 5,654,428, 5,654,435, 5,756,732, 5,164,510, 5,182,403, 5,225,565, 5,618,949, 5,627,197, 5,534,636, 5,532,261, 5,776,937, 5,529,998, 5,684,023, 5,627,181, 5,698,574, 5,220,011, 5,208,329, 5,036,092, 4,965,268, 4,921,869, 4,948,801, 5,043,443, 5,130,316, 5,254,577, 4,877,892, 4,791,207, 4,642,351, 4,665,171, 4,734,495, 4,775,752, 4,870,169, 4,668,517, 4,340,606, 4,362,866, 4,193,918, 4,000,293, 3,947,465, 4,007,168, 3,674,780, 3,686,170, 3,906,101, 3,678,040, 3,177,114, 3,141,889, 3,149,119, 3,117,122, 5,719,154, 5,254,577, 4,801,600, 4,705,799, 4,461,773, 4,243,801, 3,794,665, 3,632,577, 3,598,830, 3,513,238, 3,598,812, 3,546,241, 3,318,878, 3,322,712; the disclosures of which are incorporated herein by reference in their entirety. Preferred oxazolidinones include linezolid and eperezolid.

The description of this invention is organized into several parts. The different parts and not exclusive of each other, they all describe one invention but different aspects and applications of the invention will be emphasized and described in greater or lesser detail in the different parts of the description. One part will emphasize the methods and procedures whereby efp is used as molecular target to find, identify or characterize compounds that modulate the activity of efp, especially compounds that interfere or inhibit that activity. A subportion describes *in vitro* methods of evaluating efp and *in vivo* applications thereof. Other parts are structured similarly to the first part and conceptually should include the first part, only emphasis and methods directed to the 30S ribosomal subunit, the 50S ribosomal subunit, and the 70S ribosome are described in these other parts. Another part refers to and includes using the methods and procedures and the information in the other parts and applying it in a

novel fashion, which is to add the additional procedure of comparing the information of the first parts to with similar information about the compounds identified from steps previously identified to a similar study of the activity of those compounds on eIF5A. Another part describes compounds that are now known to have expected activity against the prokaryotic functions and systems described in the previously described parts.

The present invention is directed, in part, to methods for identifying compounds which modulate activity of efp or translation initiation complex when interacting with efp. In addition, the methods of the present invention also include, in a similar manner, identifying compounds which modulate activity of prokaryotic 30S subunit, 50S subunit, and 70S subunit of the ribosome.

Efp, as well as the other components described above, can be isolated from a natural source such as, for example, a bacteria, like *E. coli* or various other bacteria, such as, for example *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species. In addition, recombinant versions of these proteins, such as that described by M.C. Ganoza *et al., Eur. J. Biochem* 1985, vol. 146, pp. 287-294, and H. Aoki *et al. Biochimie* (1997) vol. 79, pp. 7-11, Aoki *et al., Nucleic Acids Research*, 1991 (19), pp. 6215-6220, the disclosures of which are incorporated herein by reference in their entirety, can be prepared. One skilled in the art is readily able to prepare such recombinant proteins.

In a preferred embodiment the efp can be a recombinant protein having post-translation modifications, such as, for example, those modifications selected from the group consisting of efp proteins where the lysine residues are modified. Recombinant proteins can be prepared in eukaryotic systems such as, for example, using the baculovirus expression vectors which are well known to the skilled artisan.

The preferred form of efp is the native form of the protein purified from *S. aureus, E. coli* or other pathogenic bacteria. However, according to the present invention, other forms of efp include the native form of the protein purified from various gram positive bacterial pathogens, including: *Staphylococcus aureus*; *Staphylococcus epidermidis* (A, B, C biotypes); *Staphylococcus caseolyticus*; *Staphylococcus gallinarum*; *Staphylococcus haemolyticus*; *Staphylococcus hominis*; *Staphylococcus saprophyticus*; *Streptococcus agalactiae* (group B); *Streptococcus mutans/rattus*; *Streptococcus pneumoniae*; *Streptococcus pyogenes* (group A); *Streptococcus salivarius*; *Streptococcus sanguis*; *Streptococcus sobrinus*;

Actinomyces spps.; *Arthrobacter histidinolovorans*; *Corynebacterium diptheriae*; *Clostridium difficle*; *Clostridium spps.*; *Enterococcus casseliflavus*; *Enterococcus durans*; *Enterococcus faecalis*; *Enterococcus faecium*; *Enterococcus gallinarum*; *Erysipelothrix rhusiopathiae*; *Fusobacterium spps.*; *Listeria monocytogenes*; *Prevotella spps.*; *Propionibacterium acnes*; and *Porphyromonas gingivalis*.

Still other forms of efp include the native form of the protein purified from various gram negative bacterial pathogens, including: *Acinetobacter calcoaceticus*; *Acinetobacter haemolyticus*; *Aeromonas hydrophila*; *Bordetella pertussis*; *Bordetella parapertussis*; *Bordetella bronchiseptica*; *Bacteroides fragilis*; *Bartonella bacilliformis*; *Brucella abortus*; *Brucella melitensis*; *Campylobacter fetus*; *Campylobacter jejuni*; *Chlamydia pneumoniae*; *Chlamydia psittaci*; *Chlamydia trachomatis*; *Citrobacter freundii*; *Coxiella burnetti*; *Edwardsiella tarda*; *Edwardsiella hoshinae*; *Enterobacter aerogenes*, *Enterobacter cloacae* (groups A and B); *Escherichia coli* (to include all pathogenic subtypes) *Ehrlicia spps.*; *Francisella tularensis*; *Haemophilus actinomycetemcomitans*; *Haemophilus ducreyi*; *Haemophilus haemolyticus*; *Haemophilus influenzae*; *Haemophilus parahaemolyticus*; *Haemophilus parainfluenzae*; *Hafnia alvei*; *Helicobacter pylori*; *Kingella kingae*; *Klebsiella oxytoca*; *Klebsiella pneumoniae*; *Legionella pneumophila*; *Legionella spps.*; *Morganella spps.*; *Moraxella cattarhalis*; *Neisseria gonorrhoeae*; *Neisseria meningitidis*; *Plesiomonas shigelloides*; *Proteus mirabilis*; *Proteus penneri*; *Providencia spps.*; *Pseudomonas aeruginosa*; *Pseudomonas species*; *Rickettsia prowazekii*; *Rickettsia rickettsii*; *Rickettsia tsutsugamushi*; *Rochalimaea spps.*; Salmonella subgroup 1 serotypes (to include *S. paratyphi* and *S. typhi*); Salmonella subgroups 2, 3a, 3b, 4, and 5; *Serratia marcesans*; *Serratia spps.*; *Shigella boydii*; *Shigella flexneri*; *Shigella dysenteriae*; *Shigella sonnei*; *Yersinia enterocolitica*; *Yersinia pestis*; *Yersinia pseudotuberculosis*; *Vibrio cholerae*; *Vibrio vulnificus*; and *Vibrio parahaemolyticus*.

Still other forms of efp include the native form of the protein purified from various Mycobacterial species, including: *Mycobacterium tuberculosis*; *Mycobacterium avium*; and other *Mycobacterium spps.*

Still other forms of efp include the native form of the protein purified from various Mycoplasmas (or pleuropneumonia-like organisms), including: *Mycoplasma genitalium*; *Mycoplasma pneumoniae*; and other *Mycoplasma spps.*

Still other forms of efp include the native form of the protein purified from various Treponemataceae (spiral organisms), including: *Borrelia burgdorferi*; other *Borrelia species*; *Leptospira spps.*; *Treponema pallidum.*

S. aureus efp is defined as a protein having an amino acid sequence with at least about 70% homology determined by, for example, alignment and direct one-to-one correspondence with the following protein sequence MISVNDFKTG LTISVDNAIW KVIDFQHVKP GKGSAFVRSK LRNLRTGAIQ EKTFRAGEKV EPAMIENRRM QYLYADGDNH VFMDNESFEQ TELSSDYLKE ELNYLKEGME VQIQTYEGET IGVELPKTVE LTVTETEPGI KGDTATGATK SATVETGYTL NVPLFVNEGD VLIINTGDGS YISRG (SEQ ID NO:1) and having activity in the efp activity assay described below.

E. coli efp is defined as a protein having an amino acid sequence with at least about 70% homology, determined as described above, with the following sequence MATYYSNDFRA GLKIMLDGEP YAVEASEFVK PGKGQAFARV KLRRLLTGTR VEKTFKSTDS AEGADVVDMN LTYLYNDGEF WHFMNNETFE QLSADAKAIG DNAKWLLDQA ECIVTLWNGQ PISVTPPNFV ELEIVDTDPG LKGDTAGTGG KPATLSTGAV VKVPLFVQIG EVIKVDTRSG EYVSRVK (SEQ ID NO:2) and having activity in the efp activity assay described below.

In preferred embodiments, in order to identify compounds which modulate efp activity or activity of any of the other components of the ribosome described above, *in vitro* assays are disclosed, wherein, for example, cell-free extract comprising efp (as well as any of the other components described above; 30S, 50S, and 70S) and the translation initiation complex, or components thereof, is contacted with a test compound. The contacting can take place in buffers or media well known to those skilled in the art. In addition, varying amounts of the test compound can be used as desired by the practitioner. Test compounds provided herein, including those identified by the present methods, can be formulated into pharmaceutical compositions by, for example, admixture with pharmaceutically acceptable nontoxic excipients and carriers. Test compounds which test positive can be used as antiseptic agents. Accordingly, the methods of the present invention also include a method of identifying antiseptic agents.

In some embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a solution of efp; contacting the solution containing efp with the compound; and determining whether the compound modifies activity of efp. Whether the compound modifies the activity of the efp is determined by, for example, determining whether the compound binds to efp. Binding can be determined by employing a number of art-recognized procedures.

Determining whether the compound binds to efp can be accomplished by a binding assay including, but not limited to, gel-shift mobility electrophoresis, Western blot, filter binding, and scintillation proximity assay. U.S. Patent 4,568,649, which is disclosed herein by reference in its entirety, teaches a scintillation proximity assay. Additional information regarding scintillation proximity assay systems and applications is available from Amersham Pharmacia Biotech (UK, Little Chalfont, Buckinghamshire, England HP79NA).

Determining whether the compound binds to efp can also be accomplished by measuring the intrinsic fluorescence of efp and determining whether the intrinsic fluorescence is modulated in the presence of the compound. Preferably, the intrinsic fluorescence of efp is measured as a function of the tryptophan residue(s) of efp. Preferably, fluorescence of efp is measured and compared to the fluorescence intensity of efp in the presence of the compound, wherein a decrease in fluorescence intensity indicates binding of the compound to efp. Preferred methodology is set forth in "Principles of Fluorescence Spectroscopy" by Joseph R. Lakowicz, New York, Plenum Press, 1983 (ISBN 0306412853) and "Spectrophotometry And Spectrofluorometry" by C.L. Bashford and D.A. Harris Oxford, Washington DC, IRL Press, 1987 (ISBN 0947946691), the disclosures of which are incorporated herein by reference in their entirety.

In other embodiments of the invention, the method described above further comprises determining whether the compound interfering with the function of efp is interfering with other protein(s) essential for the functioning of efp. Preferably, the other protein essential for the functioning of efp is L16 protein.

In other embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a solution of efp; contacting the solution of efp with a radiolabeled oxazolidinone, isolating or measuring the radiolabeled oxazolidinone bound to efp; contacting the compound with the radiolabeled

oxazolidinone bound to efp; and determining whether the compound displaces the radiolabeled oxazolidinone from efp. Additionally, the method may further comprise measuring the displacement of the radiolabeled oxazolidinone from efp. Preferably, determination of displacement is accomplished by comparing the amount of the detectable radiolabel in the solution prior to addition of the compound with the amount of detectable radiolabel in the solution after addition of the compound, wherein a decrease in detectable radiolabel indicates that the compound displaces the radiolabeled oxazolidinone compound from the complex. Radiolabeled competitive binding studies are described in A.H. Lin *et al. Antimicrobial Agents and Chemotherapy*, 1997, vol. 41, no. 10. pp. 2127-2131, the disclosure of which is incorporated herein by reference in its entirety. Preferably, the radiolabeled oxazolidinone compound is linezolid or eperezolid.

In some embodiments, the activity of the efp-mediated activity (or activity of any of the other ribosomal components described above) can be measured by the amount of translation initiation complex formed. One skilled in the art is readily familiar with measuring the amount of translation initiation complex formed. A compound that inhibits efp will be reflected in the amount of translation initiation complex formed *in vitro*. Preferably, the method comprises preparing a first solution of efp; preparing a second solution comprising N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3; contacting the second solution with the first solution and the compound; and determining whether the compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit, 50S subunit, any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3. Efp-mediated activity (or activity of any of the other ribosomal components described above) can be measured by measuring affinity or displacement of fMet-tRNA to said complex. A compound that inhibits the binding of fMet-tRNA to ribosomal complexes containing efp will be reflected in the amount of fMet-tRNA bound to the complex. The lower the amount of fMet-tRNA bound, the greater the inhibitory affect the test compound has. This type of affinity displacement is described by S.M. Swaney, *et al. Antimicrobial Agents and Chemotherapy* (1998) vol. 42, no. 12, pp. 3251-3255, the disclosure of which is incorporated herein by reference in its entirety. Using ordinary skills and techniques in the art the procedures in this reference can be easily adapted to the invention described herein. Preferably, the mRNA containing an AUG

sequence consists essentially of rArUrG. Preferably, efp is isolated from a natural source, such as a prokaryotic organism, preferably a bacteria including, but not limited to, *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, or an *Enterococcus* species. In all of the tests described herein, one skilled in the art can use fragments of mRNA of any length as long as the fragment comprises an AUG sequence.

In other embodiments of the invention, a method for identifying a compound which modulates the activity of efp comprises contacting a cell containing efp *in vitro* with a compound identified by the methods described above, and determining whether the compound inhibits cell growth. Alternately, the contacting can take place *in vivo*, in which an animal, such as, for example, a mammal or mouse or other suitable animal known to those skilled in the art, is contacted by administering a pharmaceutical composition comprising the test compound and pharmaceutically acceptable salt, carrier, or diluent. In addition, varying numbers of cells and concentrations of test compounds can be used. Whether the test compound increases or decreases activity of the efp is determined. In addition, whether the test compound promotes cell survival or cell death is also determined. The test compound can be administered to a mammal topically, intradermally, intravenously, intramuscularly, intraperitonally, subcutaneously, and intraosseously, or any other desired route and can be in any amount desired by the practitioner. Determination of the susceptibility of bacteria to particular compounds can be determined according to the methods described in National Committee for Clinical Laboratory Standards, **1993**, Approved standard, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd Ed., National Committee for Clinical Laboratory Standards, Villanova, PA, the disclosure of which is incorporated herein by reference in its entirety.

In other embodiments of the invention, each of the above-described methods can be applied to compositions of efp which also contain either the 30S subunit, 50S subunit, or 70S ribosome. For example, in some embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a composition or solution of efp; adding prokaryotic 30S subunit (or 50S subunit or 70S ribosome) to the solution of efp; contacting the compound with the composition or solution of efp and 30S subunit (or 50S subunit or 70S ribosome); and determining whether the compound binds to efp in association with the 30S subunit (or 50S subunit or 70S ribosome)

or whether the compound interferes with the binding of said efp and said 30S subunit (or 50S subunit or 70S ribosome). In some embodiments, determining whether the compound binds to efp in association with the 30S subunit (or 50S subunit or 70S ribosome) or whether the compound interferes with the binding of said efp and said 30S subunit (or 50S subunit or 70S ribosome) comprising determining whether the compound binds to the 30S subunit (or 50S subunit or 70S ribosome) or efp. In some embodiments, the intrinsic fluorescence of efp bound to said 30S subunit (or 50S subunit or 70S ribosome) is measured, as described above, and whether the intrinsic fluorescence is modulated by the compound is determined. Preferably, the intrinsic fluorescence of efp is measured as a function of changes in the fluorescence of the tryptophan residue(s) of efp, as described above. In addition, the above-described method may further comprise determining whether the compound interfering with the function of efp is interfering with other protein(s), such as L16 protein, essential for the functioning of efp. Determination of binding can be accomplished in the same manner as described above. In addition, competitive binding assays using a solution of efp and 30S subunit (or 50S subunit or 70S ribosome) with a radiolabeled oxazolidinone and a test compound can be performed essentially as described above.

In other embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a solution of radiolabeled efp; adding a 30S subunit (or 50S subunit or 70S ribosome) and the compound with the solution of radiolabeled efp; measure whether the 30S subunit (or 50S subunit or 70S ribosome) is bound to radiolabeled efp; and if the 30S subunit (or 50S subunit or 70S ribosome) is not bound to efp, then select the compounds which interfered with the binding thereof. Preferably, determination of binding is accomplished by employing a binding assay described above.

In other embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a first solution of efp; preparing a second solution comprising N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit (or 50S subunit or 70S ribosome), any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3; and contacting the second solution with the first solution and the compound; and determining whether the compounds allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit (or 50S subunit or 70S ribosome), any

mRNA containing the AUG sequence, and initiation factors 1, 2, and 3. Preferably, the mRNA containing an AUG sequence consists essentially of rArUrG. Preferably, efp is isolated from a natural source, such as a prokaryotic organism, such as a bacteria including, but not limited to, *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

In other embodiments of the invention, a method for identifying a compound which modulates the activity of prokaryotic efp comprises contacting a cell containing said efp and also the 30S subunit, 50S subunit or 70S ribosome with a compound identified by the previously described methods, and determining whether the compound inhibits cell growth, as described above.

In another embodiment, a method for identifying a compound which modulates the activity of prokaryotic efp comprises preparing a first solution of efp; preparing a second solution comprising 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor; contacting the second solution with the first solution and the compound; and determining whether the compound inhibits the first peptide bond reaction of a complex formed through the interaction of efp, 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor efp. Alternatively, the second solution can comprise N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, and a peptide bond donor, and it is determined whether the compound inhibits the first peptide bond reaction of a complex formed through the interaction of efp, fMet-tRNA, 30S subunit, 50S subunit, any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3. A compound that inhibits efp (or any of the other ribosomal components described above) will be reflected in the amount of first peptide bond synthesis formed *in vitro*. For example, efp allows formation of a peptide bond between N-formylmethionine and the second amino acid (or puromycin as a substitute). Addition of a compound that inhibits the action of efp will prevent formation of the peptide bond, leaving the preformed initiation complex fMet-tRNA:70S ribosome:mRNA intact. M.C. Ganoza *et al., Eur. J. Biochem* 1985, vol. 146, pp. 287-294, and/or D-G. Chung et al. Chapter 4, pp. 69-80 of Ribosomes and Protein Synthesis, A Practical Approach, edited by G. Spedding, 1990, IRL Press at Oxford University Press, Oxford, NY. and Tokyo, the disclosures of which are

incorporated herein by reference in their entirety. The first peptide bond formation can also be determined according to the methods described in Monro, *et al.*, *J. Mol. Biol.*, **1967**, *25*, 347-350, Monro, *et al.*, *Methods Enzymol.*, **1971**, *20*, 472-481, the disclosures of which are incorporated herein by reference in their entirety. Preferably, the peptide bond donor includes, but is not limited to, puromycin and analogs thereof, and any amino acyl-tRNA and analogs thereof. Preferably, efp is isolated from a natural source, such as, for example, a prokaryotic organism, preferably, a bacteria, such as, for example, *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, or an *Enterococcus* species. The above described method can also be employed to identify a compound which inhibits the first peptide bond reaction of a complex formed through the interaction of efp, 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor and efp. The above described method can also be employed to identify a compound which inhibits the first peptide bond reaction of a complex formed through the interaction of fMet-tRNA, 30S subunit, 50S subunit, any mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, and a peptide bond donor and efp. In addition, one skilled in the art can determine whether the compound, identified as described above, inhibits cell growth by contacting a cell containing efp, or any other subunits or proteins described above.

In another embodiment, a method for identifying a compound which modulates the activity of prokaryotic efp comprises contacting a cell or solution containing efp with a detectably labeled oxazolidinone compound known to bind efp under conditions whereby efp forms a complex with the oxazolidinone compound; contacting the solution or cell with an unlabeled compound; and determining whether the unlabeled compound displaces the labeled oxazolidinone compound from the complex. Preferably, the cell or solution contains an oxazolidinone compound, a compound with a substantial binding affinity for efp, 30S, 50S, or 70S, L16 protein, or other components of the ribosome. Once the cell, or cell extract, or solution containing the components, is contacted with the test compound, the ribosomal complex containing the efp (and/or the other ribosome components described above) is isolated, and it is determined to what extent the test compound has displaced oxazolidinone. There are many techniques known in the art for determining displacement. Preferably, determination of displacement is accomplished by comparing the amount of the detectable label in the cell or solution prior to addition of the unlabeled compound with the amount of

detectable label in the cell or solution after addition of unlabeled compound, wherein a decrease in detectable label indicates that the compound displaces the oxazolidinone compound from the complex. Preferably, the detectable label is a radiolabel or a fluorescent label. Radiolabeled competitive binding studies are described in A.H. Lin *et al. Antimicrobial Agents and Chemotherapy*, 1997, vol. 41, no. 10. pp. 2127-2131, the disclosure of which is incorporated herein by reference in its entirety. Fluorescent labeling is well know to the skilled artisan. Preferably, the oxazolidinone compound is linezolid or eperezolid.

In another embodiment of the invention, the test compound is further examined to determine whether it modulates the eukaryotic homolog of elongation factor p, eIF5A. eIF5A is described in Smit-McBride *et al.*, *Sequence Determination and cDNA Cloning of Eukaryotic Initiation Factor 4D, the Hypusine-containing Protein*, (1989) *J. Biol. Chem.*, vol. 264, pp. 1578-1583, the disclosure of which is incorporated herein by reference in its entirety. A method for identifying a compound which modulates the activity of prokaryotic efp but not eukaryotic eIF5A preferably comprises initially determining whether the compound modulates the activity of prokaryotic efp by any of the methods described herein; followed by the steps of preparing a first composition or solution of eIF5A; preparing a second solution comprising methionyl-tRNA (Met-tRNA), 80S ribosome, any mRNA containing an AUG sequence, initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D, and a peptide bond donor; contacting the second solution with the first solution and the compound; and determining whether the compound inhibits the first peptide bond reaction of a complex formed through the interaction of eIF5A, Met-tRNA, 80S ribosome, any mRNA containing an AUG sequence, and initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D, as described above. Preferred peptide bond donors include, but are not limited to, puromycin and analogs thereof, and any amino acyl-tRNA and analogs thereof. Preferred mRNA sequences must include rArUrG, but may include additional nucleotides. Preferably, eIF5A is isolated from a natural source, such as a eukaryotic organism, preferably a mammal.

In some embodiments of the invention, this determination is made in a manner similar to the determination for prokaryotic efp except eukaryotic eIF5A is used. Preferably, compounds which modulate prokaryotic efp but not eukaryotic eIF5A are identified as described in K. Moldave, *Eukaryotic Protein Synthesis*, (1985) *Ann. Rev. Biochem*, vol. 54, pp. 1109-1149, the disclosure of which is incorporated herein by reference in its entirety. In

addition, first peptide bond formation can be analyzed as described in Benne *et al.*, *J. Biol. Chem.*, **1978**, *253*, 3070-3087, the disclosure of which is incorporated herein by reference in its entirety.

The present invention is further directed to methods of modulating the activity of efp, 30S subunit, 50S subunit, or 70S subunit, L16 protein, or ribosomal subunits containing any of the same, or cells or cell preparations (including cell lysates) containing any of the same by contacting any of the above-described samples with an oxazolidinone compound. Contacting can be *in vitro* or *in vivo* by any of the routes of administration described above. The oxazolidinone can be formulated as described above into a pharmaceutical composition.

The invention is further illustrated by way of the following examples which are intended to elucidate the invention. These examples are not intended, nor are they to be construed, as limiting the scope of the disclosure.

EXAMPLES

Example 1: Assay to identify compounds that bind to efp by displacing a bound radiolabeled oxazolidinone

Materials. Unlabeled PNU100592 10 mM, a nitrocellulose membrane such as, Millipore Immobilon-P^{SQ} membrane (pre-treated with 70% ethanol and rinsed with deionized water).

The purification of the native efp protein has been described previously. *Aoki et al. Biochime* 1997, vol. 79, pp. 7-11. "Molecular characterization of the prokaryotic efp gene product involved in a peptidyl transferase reaction" and/or D-G. Chung et al. Chapter 4, pp. 69-80 of Ribosomes and Protein Synthesis, A Practical Approach, edited by G. Spedding, 1990, IRL Press at Oxford University Press, Oxford, NY. and Tokyo, both articles incorporated here by reference. Purification of efp can also be performed according to the methods of Aoki, *et al.*, *Nuc. Acids Res.*, **1991**, *19*, 6215-6220, which is incorporated herein by reference in its entirety. Purification of efp can also be accomplished with an immunoaffinity column using antibodies directed against *E. coli* and *S. aureus* efp. Recombinant efp from *S. aureus* or *E. coli* is preferably expressed as a Histag fusion protein,

expressed in *E. coli*, and purified via affinity column chromatography using Ni-NTA-Sepharose. Bound efp is preferably eluted in an imidazole gradient.

An efp reaction mixture (final concentrations in a reaction mixture of 100 μl) is prepared containing TKM Buffer (50 mM Tris, pH 7.5, 200 mM KCl, 5 mM magnesium acetate), Elongation factor P (EFP) 0.192 mg/ml or 9 μM, radiolabeled C^{14}-linezolid or C^{14}-eperezolid 10 μM (stock 24.3 mCi/mmol, 59.32 μCi/mg) or H^3-eperezolid, purified *E. coli* or *S. aureus* elongation factor P and the compound to be tested. The reaction is incubated at room temperature for 30 min. The reaction mixture is then carefully deposited in the center of the membrane disk on a vacuum manifold with very low vacuum setting and washed with 5 ml TKM buffer. The amount of membrane-bound EFP thus collected is measured by liquid scintillation spectrometry.

Efp function can be assayed as previously described M.C. Ganoza *et al., Eur. J. Biochem* 1985, vol. 146, pp. 287-294, and/or D-G. Chung et al. Chapter 4, pp. 69-80 of Ribosomes and Protein Synthesis, A Practical Approach, edited by G. Spedding, 1990, IRL Press at Oxford University Press, Oxford, NY. and Tokyo, both articles incorporated by reference.

Purification of Ribosomal Subunits without Efp

S. aureus cells (50 g wet weight) were resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mg/ml lysostaphin, 10,000 U Dnase I (Boehringer Mannheim, Indianapolis, IN.)) and incubated for 1 h in a 37C water bath. Beta-mercaptoethanol was added to a final concentration of 5 mM, and the lysed cells were centrifuged at 10,000 x g for 10 min to remove unbroken cells and cell fragments. The supernatant was centrifuged at 30,000 x g and the resulting supernatant was centrifuged at 100,000 x g for 16 h to pellet the ribosomes. The ribosome pellet was resuspended in Buffer B (20 mM Tris-HCl, pH 7.4, 1 M NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT) and again centrifuged at 100,000 x g for 16 h. The pellet was resuspended in Buffer A (60 mM NH_4Cl), applied to linear 5-40% (w/v) sucrose gradients prepared in Buffer A, and centrifuged for 16 h in a Beckman SW28 rotor. Gradients were fractionated, the 70S ribosomes were pooled, pelleted at 300,000 x g for 5 h, and resuspended in Buffer A before storing at -80C.

One hundred grams of *E. coli* MRE600 grown in NS87 medium plus 1% yeast extract were washed with Buffer LM (10mM Tris-HCl, pH 7.8, 10 mM NH_4Cl, 1 mM $Mg(OAc)_2$, 1mM DTT), ground with two weights of alumina for 20 min and the paste extracted with 100 ml of Buffer LM containing 4 μg Dnase. An S30 fraction was prepared by centrifuging the suspension for 20 min at 20,000 x g and recentrifuging the supernatant for 30 min at 30,000 x g. The resulting supernatant fraction (S30) was adjusted to contain a final NH_4Cl concentration of 1.0 M by slowly adding solid NH_4Cl. The salt-washed ribosomes were pelleted by centrifuging the S30 for 4 hr at 150,000 x g. The washed ribosomes thus obtained were suspended in a small volume of Buffer LM and subjected to centrifugation in a 10-30% sucrose gradient in Buffer LM for 16 hr at 18,000 rpm in an SW28 rotor. The pooled fractions containing 30S and 50S subunits and the 70S tight couples were collected by pelleting at 100,000 x g for 24 hr and resuspended in Buffer LM. The subunits were further purified by an additional round of sucrose gradient centrifugation. The purity of the subunits was verified by RNA analysis.

Purification of Ribosomal Subunits with Efp

S. aureus cells (50 g wet weight) were resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mg/ml lysostaphin, 10,000 U Dnase I (Boehringer Mannheim, Indianapolis, IN.)) and incubated for 1 h in a 37C water bath. Beta-mercaptoethanol was added to a final concentration of 5 mM, and the lysed cells were centrifuged at 10,000 x g for 10 min to remove unbroken cells and cell fragments. The supernatant was centrifuged at 30,000 x g and the resulting supernatant was centrifuged at 100,000 x g for 16 h to pellet the ribosomes. The ribosome pellet was resuspended in Buffer A (60 mM NH_4Cl) and again centrifuged at 100,000 x g for 16 h. The pellet was resuspended in Buffer A, applied to linear 5-40% (w/v) sucrose gradients prepared in Buffer A, and centrifuged for 16 h in a Beckman SW28 rotor. Gradients were fractionated, the 70S ribosomes were pooled, pelleted at 300,000 x g for 5 h, and resuspended in Buffer A before storing at -80C.

One hundred grams of *E. coli* MRE600 grown in NS87 medium plus 1% yeast extract were washed with Buffer LM (10mM Tris-HCl, pH 7.8, 10 mM NH_4Cl, 1 mM $Mg(OAc)_2$, 1mM DTT), ground with two weights of alumina for 20 min and the paste

extracted with 100 ml of Buffer LM containing 4 μg Dnase. An S30 fraction was prepared by centrifuging the suspension for 20 min at 20,000 x g and recentrifuging the supernatant for 30 min at 30,000 x g. The resulting supernatant fraction (S30) was centrifuged for 4 hr at 150,000 x g. The peletted ribosomes thus obtained were suspended in a small volume of Buffer LM and subjected to centrifugation in a 10-30% sucrose gradient in Buffer LM for 16 hr at 18,000 rpm in an SW28 rotor. The pooled fractions containing 30S and 50S subunits and the 70S tight couples were collected by pelleting at 100,000 x g for 24 hr and resuspended in Buffer LM. The subunits were further purified by an additional round of sucrose gradient centrifugation. The purity of the subunits was verified by RNA analysis.

Example 2: Elongation Factor P (efp) Tryptophan Fluorescence

Fluorescence measurements are carried out suing an ISS Spectroflurometer. The sample holder in the instrument is maintained at 26C using recirculated water from a constant temperature bath. Buffer solution consisting of 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl, and 0.0001-0/001% Tween-20 is prepared. Other buffer systems such as HEPES, MOPS, Na-acetate, Na-phosphate may be used instead of Tris-HCl. The addition of Tween-20 or other nonionic detergent is necessary to prevent absorption of EFP to the walls of quartz cuvette. The buffer is passed through a 0.2 μm filter and degassed before use. Two milliliters of buffer are pipeted into a quartz cuvette (1 cm pathlength) containing a Teflon stir-bar. The quartz cuvette is placed into the temperature controlled sample holder of the spectrofluorometer. A solution of EFP protein (600 nM to 2.4 μM final protein concentration) is prepared by adding a specific volume of a stock solution of EFP protein to the cuvette containing 2 ml of buffer. A typical experiment uses 600 nM EFP, but data may be obtained with higher concentrations of protein. The cuvette containing the EFP solution is allowed to equilibrate with stirring for 10-15 minutes at 26C in the fluorometer. This time is required for equilibrium to occur between protein in solution and protein bound to the cuvette, and for the protein solution to reach the controlled temperature. A baseline fluorescence reading of EFP is obtained before adding any drug. The fluorescence of the single tryptophan residue (S. Aureus EFP) or three tryptophan residues (E. Coli EFP) are measured using an excitation wavelength of 295 nm and an emission wavelength of 330 nm. Slit widths for excitation and emission wavelengths are 1 mm. Excitation wavelengths

between 270-300 nm, emission wavelengths between 310-350 mm, and other slit widths may be used to measure the tryptophan fluorescence of EFP protein. A stock solution of drug (oxazolidinone or other test substance) is prepared in 100% DMSO. A typical experiment uses a 2 mM solution of drug in 100% DMSO. The drug is added in small increments (0.5-5.0 μl) and the fluorescence intensity at 330 nm is recorded after each addition. The final concentration of drug (oxazolidinone) is calculated after each drug addition. In a typical experiment, a total volume of 42 μl of 2 mM drug solution is added to give a final concentration of 42 μMolar oxazolidinone. The intensity of the tryptophan fluorescence is plotted vs drug concentration and the data is fit the following equation:

$$F = \left[\frac{(F_0-(F_0-F_{00}))\times I}{I+K_a}\right]^{-IXI}$$

where F is fluorescence intensity at 330 nm, F_0 is initial fluorescence without drug, F_{00} is the fluorescence intensity where the protein is saturated with drug, I is the drug concentration, K_d is the dissociation constant for the drug-protein interaction, and I is the correction factor for the inner filter effect. The oxazolidinones exhibit significant absorbance at the excitation wavelength (295 nm) so a correction for the inner filter effect of the drug is necessary. Goodness of fit of the experimental data tot he theoretical curve is evaluated using the residual sum of squares.

Example 3: Screening Assay

Radio-labeled compounds: [^{14}C]eperezolid (59.32 μCi/mg, 23.4 mCi/mmol), [^{14}C]linezolid (63.9 μCi/mg), and [^{3}H]eperezolid were synthesized at Pharmacia and Upjohn, Inc using standard technology. The binding studies were performed in microcentrifuge tubes that contained a total of 100 μl of reaction mixture which included 4-28 A_{260} units of ribosomes containing efp, 1 to 100 μM of radio-labeled compound with either 1 μl of DMSO or an excess amount (100- to 1000-fold) of unlabeled compound, 50 mM Tris-HCl, pH 7.5, 5 mM $Mg(OAc)_2$, 200 mM KCl. All other ingredients were mixed together before the addition of ribosome. Ribosomes containing efp were prepared as described above. Alternately, ribosomes without efp can be prepared as described above and purified efp can be added. The reaction mixture was allowed to incubate at 25C for 10 min and was terminated by the

addition of 50 μl ice-cold ethanol. After incubation at 4C for 30-60 min, the suspension was centrifuged at full speed in an Eppendorf for 20 min. The supernatant was then carefully removed and the radioactivity in the pellet measured. Nonspecific binding is defined as the number of dpm in the presence of an excess amount of unlabeled compound. The specific binding is determined by subtracting the nonspecific from the total binding.

Once the compounds that bind to either the 50S or 70S bacterial ribosomes have been identified, they will be screened for their ability to inhibit the formation of the translation initiation complex in the translation initiation complex assay.

The identification of compounds that compete with the binding of radiolabeled oxazolidinones to 50S or 70S bacterial ribosomes and inhibit the formation of the translation initiation complex.

Example 4: Initiation Complex Assay

S. aureus or *E. coli* 70S ribosomes, without efp, (10 pmol) were incubated with 9 pmoles [^{3}H]fmet-tRNA in duplicate 100 μl reactions containing 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 30 mM NH_4Cl, 1 mM DTT, various concentrations of the test compound and 100 pmol of the oligoribonucleotide (5'-rGGGAAUUCGGAGGUUUAAAAAUGGGUAAA-3'; SEQ ID NO:3). Duplicate reactions were incubated at 37C for 10 min and stopped by the addition of 2 ml of cold Buffer A (10 mM Tris-HCl, pH 7.4, 30 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT). Complexes were filtered through Millipore filters (0.45μ), washed with 50 μl of Buffer A and were counted after the addition of scintillation fluid. Compounds that inhibit the formation of the initiation complex would result in a decrease in the amount of [^{3}H]fmet-tRNA bound to the complex and trapped on the filter. A similar assay can be employed using 70S ribosomes in the presence of efp by purifying the ribosome subunits and efp as described above.

Example 5: Efp-Mediated First Peptide Bond Assay

Native efp purified from bacteria by the method of Aoki *et al.* is assayed in a total volume of 50 μl containing 1 ng - 1 mg of efp, 50 pmoles 70S ribosomes, 150 pmoles AUG or mRNA containing AUG, 5 pmoles radiolabeled fMet-tRNA, 55 mM NH_4Cl, 8 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.4. The ribosomes are incubated for 5 min at 30C, and the

AUG and radiolabeled fMet-tRNA are added. After a 30 min incubation at 30C, the efp source and puromycin (or suitable tRNA) are preincubated with each other for 1 min at 30C. The reaction is started by mixing the efp/puromycin solution with the ribosome/AUG/fMet-tRNA solution, followed by a 30 min incubation at 30C. The reaction is stopped by adding 100 µl of 1 M potassium phosphate buffer, pH 6.0. The f-met-puromycin (or fMet-amino acid) is extracted with 1 ml of ethyl acetate, added to the scintillation cocktail, and counted in a scintillation counter.

Some of the preferred embodiments of the invention described above are outlined below and include, but are not limited to, the following claimed embodiments. As those skilled in the art will appreciate, numerous changes and modifications may be made to the preferred embodiments of the invention without departing from the spirit of the invention. It is intended that all such variations fall within the scope of the invention.

WHAT IS CLAIMED IS:

1. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a compound; and

(b) determining whether said compound modifies activity of efp.

2. The method of claim 1 wherein step (b) comprises determining whether said compound binds to efp.

3. The method of claim 2 wherein step (b) is determined by measuring the intrinsic fluorescence of efp and determining whether said intrinsic fluorescence is modulated by said binding.

4. The method of claim 3 wherein said intrinsic fluorescence of efp is measured as a function of the tryptophan residue(s) of efp.

5. The method of claim 4 wherein said fluorescence of efp is measured and compared to the fluorescence intensity of efp in the presence of the compound, wherein a decrease in fluorescence intensity indicates binding of efp.

6. The method of claim 1 further comprising step:

(c) determining whether said compound interfering with the function of efp is interfering with other protein(s) essential for the functioning of efp.

7. The method of claim 6 wherein said other protein essential for the functioning of efp is L16 protein.

8. The method of claim 2 wherein step (b) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

9. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a radiolabeled oxazolidinone;

(b) isolating or measuring said radiolabeled oxazolidinone bound to efp;

(c) contacting a compound with said radiolabeled oxazolidinone bound to efp; and

(d) determining whether said compound displaces said radiolabeled oxazolidinone from efp.

10. The method of claim 9 further comprising the step:

(e) measuring the displacement of the radiolabeled oxazolidinone from efp.

11. The method of claim 9 wherein step (d) is determined by comparatively measuring radioactivity of efp bound to said radiolabeled oxazolidinone with the radioactivity of efp in the presence of the compound.

12. The method of claim 10 wherein said radiolabeled oxazolidinone compound is linezolid or eperezolid.

13. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 to form a second composition;

(b) contacting said second composition with a compound; and

(c) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3.

14. The method of claim 13 wherein said mRNA containing an AUG sequence consists essentially of rArUrG.

15. The method of any one of claims 1, 9 and 13 wherein efp is isolated from a natural source.

16. The method of claim 15 wherein said natural source is a prokaryotic organism.

17. The method of claim 16 wherein said prokaryotic organism is a bacteria.

18. The method of claim 17 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

19. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell containing efp with a compound identified in claim 9 or 13; and

(b) determining whether said compound inhibits cell growth.

20. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting radiolabeled oxazolidinone bound to efp with a compound; and

(b) determining whether said compound displaces said radiolabeled oxazolidinone from efp.

21. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a composition comprising efp, N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 with a compound; and

(b) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3.

22. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 30S subunit to form a composition;

(b) contacting said composition with a compound; and

(c) determining whether said compound binds to efp in association with said 30S subunit or whether said compound interferes with the binding of efp and said 30S subunit.

23. The method of claim 22 wherein step (c) comprises determining whether said compound binds to said 30S subunit.

24. The method of claim 22 wherein step (c) comprises determining whether said compound binds to efp.

25. The method of claim 23 or 24 wherein step (c) is determined by measuring the intrinsic fluorescence of efp bound to said 30S subunit and determining whether said intrinsic fluorescence is modulated by said compound.

26. The method of claim 25 wherein said intrinsic fluorescence of efp is measured as a function of changes in the fluorescence of the tryptophan residue(s) of efp.

27. The method of claim 26 wherein said fluorescence of efp is measured and compared to the fluorescence intensity of efp in the presence of the compound, wherein a decrease in fluorescence intensity indicates binding of efp.

28. The method of claim 22 further comprising step (d), determining whether said compound interfering with the function of efp is interfering with other protein(s) essential for the functioning of efp.

29. The method of claim 28 wherein said other protein essential for the functioning of efp is L16 protein.

30. The method of claim 23 or 24 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

31. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 30S subunit to form a composition;

(b) contacting said composition with a radiolabeled oxazolidinone;

(c) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 30S subunit;

(d) contacting said radiolabeled oxazolidinone bound to said efp and 30S subunit with a compound; and

(e) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 30S subunit.

32. The method of claim 31 further comprising the step:

(f) measuring the displacement of the radiolabeled oxazolidinone from efp and said 30S subunit.

33. The method of claim 31 wherein step (d) is determined by comparatively measuring radioactivity of efp and 30S subunit bound to said radiolabeled oxazolidinone with radioactivity of efp and 30S subunit in the presence of the compound.

34. The method of claim 33 wherein said radiolabeled oxazolidinone compound in linezolid or eperezolid.

35. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting radiolabeled efp with prokaryotic 30S subunit to form a composition;

(b) contacting said composition with a compound;

(c) measuring whether said 30S subunit is bound to radiolabeled efp; and

(d) if said 30S subunit is not bound to efp, then select the compounds which interfered with said binding.

36. The method of embodiment 35 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

37. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 to form a second composition;

(b) contacting said second composition with said compound; and

(c) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

38. The method of claim 37 wherein said mRNA containing an AUG sequence consists essentially of rArUrG.

39. The method of any one of claims 22, 31, 35 or 37 wherein efp is isolated from a natural source.

40. The method of claim 39 wherein said natural source is a prokaryotic organism.

41. The method of claim 40 wherein said prokaryotic organism is a bacteria.

42. The method of claim 41 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

43. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell comprising efp with a compound identified in claim 31, 35 or 37; and

(b) determining whether said compound inhibits cell growth.

44. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 30S subunit and a radiolabeled oxazolidinone;

(b) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 30S subunit;

(c) contacting said radiolabeled oxazolidinone bound to said efp and 30S subunit with a compound; and

(d) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 30S subunit.

45. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a composition comprising efp, N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 with a compound; and

(b) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 30S subunit, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

46. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 50S subunit to form a composition;

(b) contacting said composition with a compound; and

(c) determining whether said compound binds to efp in association with said 50S subunit or whether said compound interferes with the binding of efp and said 50S subunit.

47. The method of claim46 wherein step (c) comprises determining whether said compound binds to said 50S subunit.

48. The method of claim 46 wherein step (c) comprises determining whether said compound binds to efp.

49. The method of claim 47 or 48 wherein step (c) is determined by measuring the intrinsic fluorescence of efp bound to said 50S subunit and determining whether said intrinsic fluorescence is modulated by said compound.

50. The method of claim 49 wherein said intrinsic fluorescence of efp is measured as a function of changes in the fluorescence of the tryptophan residue(s) of efp.

51. The method of claim 50 wherein said fluorescence of efp is measured and compared to the fluorescence intensity of efp in the presence of the compound, wherein a decrease in fluorescence intensity indicates binding of efp.

52. The method of claim 46 further comprising step (d), determining whether said compound interfering with the function of efp is interfering with other protein(s) essential for the functioning of efp.

53. The method of claim 52 wherein said other protein essential for the functioning of efp is L16 protein.

54. The method of claim 47 or 48 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

55. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 50S subunit to form a composition;

(b) contacting said composition with a radiolabeled oxazolidinone;

(c) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 50S subunit;

(d) contacting said radiolabeled oxazolidinone bound to said efp and 50S subunit with a compound; and

(e) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 50S subunit.

56. The method of claim 55 further comprising the step:

(f) measuring the displacement of the radiolabeled oxazolidinone from efp and said 50S subunit.

57. The method of claim 55 wherein step (d) is determined by comparatively measuring radioactivity of efp and 50S subunit bound to said radiolabeled oxazolidinone with radioactivity of efp and 50S subunit in the presence of the compound.

58. The method of claim 57 wherein said radiolabeled oxazolidinone compound in linezolid or eperezolid.

59. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting radiolabeled efp with prokaryotic 50S subunit to form a composition;

(b) contacting said composition with a compound;

(c) measuring whether said 50S subunit is bound to radiolabeled efp; and

(d) if said 50S subunit is not bound to efp, then select the compounds which interfered with said binding.

60. The method of claim 59 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

61. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising N-formylmethionyl-tRNA (fMet-tRNA), 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 to form a second composition;

(b) contacting said second composition with said compound; and

(c) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 50S subunit, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

62. The method of claim 61 wherein said mRNA containing an AUG sequence consists essentially of rArUrG.

63. The method of any one of claims 46, 55, 59 or 61 wherein efp is isolated from a natural source.

64. The method of claim 63 wherein said natural source is a prokaryotic organism.

65. The method of claim 64 wherein said prokaryotic organism is a bacteria.

66. The method of claim 65 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

67. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell containing said efp with a compound identified in claim 55, 59 or 61; and

(b) determining whether said compound inhibits cell growth.

68. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 50S subunit and a radiolabeled oxazolidinone;

(b) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 50S subunit;

(c) contacting said radiolabeled oxazolidinone bound to said efp and 50S subunit with a compound; and

(d) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 50S subunit.

69. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a composition comprising efp, N-formylmethionyl-tRNA (fMet-tRNA), 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 with a compound; and

(b) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 50S subunit, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

70. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 70S ribosome to form a composition;

(b) contacting said composition with a compound; and

(c) determining whether said compound binds to efp in association with said 70S ribosome or whether said compound interferes with the binding of efp and said 70S ribosome.

71. The method of claim 70 wherein step (c) comprises determining whether said compound binds to said 70S ribosome.

72. The method of claim 70 wherein step (c) comprises determining whether said compound binds to efp.

73. The method of claim 71 or 72 wherein step (c) is determined by measuring the intrinsic fluorescence of efp bound to said 70S ribosome and determining whether said intrinsic fluorescence is modulated by said compound.

74. The method of claim 73 wherein said intrinsic fluorescence of efp is measured as a function of changes in the fluorescence of the tryptophan residue(s) of efp.

75. The method of claim 74 wherein said fluorescence of efp is measured and compared to the fluorescence intensity of efp in the presence of the compound, wherein a decrease in fluorescence intensity indicates binding of efp.

76. The method of claim 70 further comprising step (d), determining whether said compound interfering with the function of efp is interfering with other protein(s) essential for the functioning of efp.

77. The method of claim 76 wherein said other protein essential for the functioning of efp is L16 protein.

78. The method of claim 71 or 72 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

79. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with prokaryotic 70S ribosome to form a composition;

(b) contacting said composition with a radiolabeled oxazolidinone;

(c) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 70S ribosome;

(d) contacting said radiolabeled oxazolidinone bound to said efp and 70S ribosome with a compound; and

(e) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 70S ribosome.

80. The method of claim 79 further comprising the step:

(f) measuring the displacement of the radiolabeled oxazolidinone from efp and said 70S ribosome.

81. The method of claim 79 wherein step (d) is determined by comparatively measuring radioactivity of efp and 70S ribosome bound to said radiolabeled oxazolidinone with radioactivity of efp and 70S ribosome in the presence of the compound.

82. The method of claim 81 wherein said radiolabeled oxazolidinone compound in linezolid or eperezolid.

83. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting radiolabeled efp with prokaryotic 70S ribosome to form a composition;

(b) contacting said composition with a compound;

(c) measuring whether said 70S ribosome is bound to radiolabeled efp; and

(d) if said 70S ribosome is not bound to efp, then select the compounds which interfered with said binding.

84. The method of claim 83 wherein step (c) comprises a binding assay selected from the group consisting of gel electrophoresis, Western blot, filter binding, and scintillation proximity assay.

85. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising N-formylmethionyl-tRNA (fMet-tRNA), 70S ribosome, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 to form a second composition;

(b) contacting said second composition with said compound; and

(c) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 70S ribosome, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

86. The method of claim 85 wherein said mRNA containing an AUG sequence consists essentially of rArUrG.

87. The method of any one of claims 70, 79, 83 or 86 wherein efp is isolated from a natural source.

88. The method of claim 87 wherein said natural source is a prokaryotic organism.

89. The method of claim 88 wherein said prokaryotic organism is a bacteria.

90. The method of claim 89 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

91. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell comprising efp with a compound identified in claim 79, 83 or 85; and

(b) determining whether said compound inhibits cell growth.

92. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with 70S ribosome and a radiolabeled oxazolidinone;

(b) isolating or measuring said radiolabeled oxazolidinone bound to said efp and 70S ribosome;

(c) contacting said radiolabeled oxazolidinone bound to said efp and 70S ribosome with a compound; and

(d) determining whether said compound displaces said radiolabeled oxazolidinone from efp and said 70S ribosome.

93. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a composition comprising efp, N-formylmethionyl-tRNA (fMet-tRNA), 70S ribosome, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3 with a compound; and

(b) determining whether said compound allows fMet-tRNA to bind to a complex formed through the interaction of efp, 70S ribosome, an mRNA containing the AUG sequence, and initiation factors 1, 2, and 3.

94. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising either 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor to form a second composition;

(b) contacting said second composition with a compound; and

(c) determining whether said compound inhibits the first peptide bond reaction.

95. The method of claim 94 wherein the peptide bond donor is either puromycin or a puromycin analog.

96. The method of claim 94 wherein the peptide bond donor is an amino acyl-tRNA or an analog of amino acyl-tRNA.

97. A method for identifying a compound which inhibits the first peptide bond reaction of a complex formed through the interaction of efp, 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor comprising the steps of:

(a) contacting efp with a composition comprising 50S subunit or 70S ribosome, a tRNA fragment comprising CACCA-radiolabeled amino acid, and a peptide bond donor to form a second composition;

(b) contacting said second composition with a compound; and

(c) determining whether said compounds inhibits the first peptide bond reaction.

98. The method of claim 97 wherein said peptide bond donor is either puromycin or a puromycin analog.

99. The method of claim 97 wherein said peptide bond donor is an amino acyl-tRNA or an analog of amino acyl-tRNA.

100. The method of claims 94 or 97 wherein said efp is isolated from a natural source.

101. The method of claim 100 wherein said natural source is a prokaryotic organism.

102. The method of claim 101 wherein said prokaryotic organism is bacteria.

103. The method of claim 102 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

104. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting efp with a composition comprising N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, and a peptide bond donor to form a second composition;

(b) contacting said second composition with a compound; and

(c) determining whether said compound inhibits the first peptide bond reaction formed by the complex containing N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, peptide bond donor and efp.

105. The method of claim 104 wherein the peptide bond donor is either puromycin or a puromycin analog.

106. The method of claim 105 wherein said mRNA sequence is rArUrG.

107. The method of claim 106 wherein the peptide bond donor is an amino acyl-tRNA or an analog of amino acyl-tRNA.

108. The method of claim 107 wherein said mRNA sequence is rArUrG.

109. A method for identifying a compound which inhibits the first peptide bond reaction of a complex formed through the interaction of efp, N-formylmethionyl-tRNA (fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, and a peptide bond donor comprising the steps of:

(a) contacting efp with a composition comprising fMet-tRNA), 30S subunit, 50S subunit, an mRNA containing an AUG sequence, and initiation factors 1, 2, and 3, and a peptide bond donor to form a second composition;

(b) contacting said second composition with a compound; and

(c) determining whether said compound inhibits the first peptide bond reaction of the complex of fMet-tRNA, 30S subunit, 50S subunit, an mRNA containing an AUG sequence, initiation factors 1, 2, and 3, peptide bond donor, and efp.

110. The method of claim 109 wherein said peptide bond donor is either puromycin or a puromycin analog.

111. The method of claim 110 wherein said mRNA sequence is rArUrG.

112. The method of claim 109 wherein said peptide bond donor is an amino acyl-tRNA or an analog of amino acyl-tRNA.

113. The method of claim 112 wherein said mRNA sequence is rArUrG.

114. The method of claims 94, 97, 104 or 109 wherein said efp is isolated from a natural source.

115. The method of claim 114 wherein said natural source is a prokaryotic organism.

116. The method of claim 115 wherein said prokaryotic organism is bacteria.

117. The method of claim 116 wherein said bacteria is selected from the group consisting of *E. coli*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and an *Enterococcus* species.

118. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell containing said efp with a compound identified in claim 94, 97, 104 or 109; and

(b) determining whether said compound inhibits cell growth.

119. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell or composition containing efp with a detectably labeled oxazolidinone compound known to bind efp under conditions whereby efp forms a complex with said oxazolidinone compound;

(b) contacting said composition or cell with an unlabeled compound; and

(c) determining whether said unlabeled compound displaces said labeled oxazolidinone compound from said complex.

120. The method of claim 119 wherein said displacement in step (c) is determined by comparing the amount of said detectable label in said cell or composition prior to addition of said unlabeled compound with the amount of said detectable label in said cell or composition after addition of said unlabeled compound, wherein a decrease in detectable label indicates said compound displaces said oxazolidinone compound from said complex.

121. The method of claim 120 wherein said detectable label is a radiolabel or a fluorescent label.

122. The method of claim 121 wherein said oxazolidinone compound is linezolid or eperezolid.

123. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) comprising the steps of:

(a) contacting a cell or composition containing efp and a detectably labeled oxazolidinone compound known to bind efp under conditions whereby efp forms a complex with said oxazolidinone compound with an unlabeled compound; and

(b) determining whether said unlabeled compound displaces said labeled oxazolidinone compound from said complex.

124. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) but not eukaryotic eIF5A comprising the steps of:

(a) determining whether said compound modulates the activity of prokaryotic efp by the method of any one of claims 1, 9, 13, 22, 31, 35, 37, 46, 55, 59, 61, 67, 70, 79, 83, 85, 94, 97, 104 or 119;

(b) contacting eIF5A with a composition comprising methionyl-tRNA (Met-tRNA), 80S ribosome, an mRNA containing an AUG sequence, initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D, and a peptide bond donor to form a second composition;

(c) contacting said second composition with a compound; and

(d) determining whether said compound inhibits the first peptide bond reaction of a complex formed through the interaction of eIF5A, Met-tRNA, 80S ribosome, an mRNA containing an AUG sequence, and initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D.

125. The method of claim 124 wherein said peptide bond donor is either puromycin or a puromycin analog.

126. The method of claim 125 wherein said mRNA sequence is rArUrG.

127. The method of claim 124 wherein said peptide bond donor is an amino acyl-tRNA or an analog of amino acyl-tRNA.

128. The method of claim 127 wherein said mRNA sequence is rArUrG.

129. The method of claim 124 wherein efp is isolated from a natural source.

130. The method of claim 129 wherein said natural source is a eukaryotic organism.

131. The method of claim 130 wherein said eukaryotic organism is a mammal.

132. A method for identifying a compound which modulates the activity of prokaryotic elongation factor p (efp) but not eukaryotic eIF5A comprising the steps of:

(a) determining whether said compound modulates the activity of prokaryotic efp by the method of any one of claims 1, 9, 13, 22, 31, 35, 37, 46, 55, 59, 61, 67, 70, 79, 83, 85, 94, 97, 104 or 119;

(b) contacting a composition comprising eIF5A, methionyl-tRNA (Met-tRNA), 80S ribosome, an mRNA containing an AUG sequence, initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D, and a peptide bond donor with a compound; and

(c) determining whether said compound inhibits the first peptide bond reaction of a complex formed through the interaction of eIF5A, Met-tRNA, 80S ribosome, an mRNA containing an AUG sequence, and initiation factors eIF-2, eIF-3, eIF-5, eIF-4C, eIF-4D.

133. A method of modulating the activity of prokaryotic efp comprising contacting efp or a cell or cell preparation containing efp with an oxazolidinone compound.

134. A method of modulating the activity of 30S subunit comprising contacting 30S subunit in association with efp with an oxazolidinone compound.

135. A method of claim 134 wherein said 30S subunit in association with efp is in a cell or cell preparation.

136. A method of modulating the activity of procaryotic 50S subunit comprising contacting said 50S subunit in association with efp with an oxazolidinone compound.

137. A method of claim 136 wherein said 50S subunit in association with efp is in a cell or cell preparation.

138. A method of modulating the activity of 70S ribosome comprising contacting said 70S ribosome in association with efp with an oxazolidinone compound.

139. A method of claim 138 wherein said 70S ribosome in association with efp is in a cell or cell preparation.

140. A method of modulating the activity of L16 protein comprising contacting said L16 protein in association with efp with an oxazolidinone compound.

141. A method of claim 140 wherein said L16 protein in association with efp is in a cell or cell preparation.

ABSTRACT OF THE DISCLOSURE

Disclosed are novel methods of using elongation factor p (efp) and related constituents of ribosomal complexes which comprise efp, the 50S ribosomal subunit, the 30S ribosomal subunit, the 70S initiation complex, and related proteins, cofactors and enzymes. Methods of identifying compounds which modulate prokaryotic elongation factor p and modify cell function are described. Both *in vitro* and *in vivo* methods for identifying compounds which modulate such constituents and affect cell function are described. Such identified compounds, including various antibiotics, which specifically affect cell growth, methods of treating various disorders with such compounds, and antiseptics containing such compounds are described. The present invention is also directed to methods and compounds that modulate prokaryotic elongation factor p.

DOCKET NO. PUJ-0041 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Keith R. Marotti, Roger A. Poorman, Peter Andrew Wells, Dean L. Shinaberger

Group Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

For: ELONGATION FACTOR P (EFP) AND ASSAYS AND ANTIMICROBIAL TREATMENTS RELATED TO THE SAME

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☒ is attached hereto.

☐ was filed on _____ as Serial No. _____ .

☐ said application having been amended on _____ .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Suzanne E. Miller Reg. No. 32,279

Paul K. Legaard Reg. No. 38,534

Address all telephone calls and correspondence to:

Paul K. Legaard
WOODCOCK WASHBURN KURTZ
MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone No.: **(215) 568-3100**
Facsimile No.: **(215) 568-3439**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: Keith R. Marotti	
Mailing Address: 2707 Lomond Drive Kalamazoo, Michigan 49008	**Signature** **Date of Signature:** _____
City/State of Actual Residence: Kalamazoo, Michigan 49008	**Citizenship:** United States

Name: Roger A. Poorman	
Mailing Address: 328 South Kendall, Apartment #31 Kalamazoo, Michigan 49007	**Signature** **Date of Signature:** _____
City/State of Actual Residence: Kalamazoo, Michigan 49007	**Citizenship:** United States

Name: Peter Andrew Wells	
Mailing Address: 1064 Josiane Kalamazoo, Michigan 49009	**Signature** **Date of Signature:** _____
City/State of Actual Residence: Kalamazoo, Michigan 49009	**Citizenship:** United States

Name: Dean L. Shinabarger	
Mailing Address: 10810 Cora Drive Portage, Michigan 49002	**Signature** **Date of Signature:** _____
City/State of Actual Residence: Portage, Michigan 49002	**Citizenship:** United States

Name:	
Mailing Address:	**Signature** **Date of Signature:** _____
City/State of Actual Residence:	**Citizenship:** _____

Name:	
Mailing Address:	**Signature** **Date of Signature:** _____
City/State of Actual Residence:	**Citizenship:** _____

SEQUENCE LISTING

<110> Marotti, Keith R.
Poorman, Roger A.
Wells, Peter Andrew
Shinabarger, Dean L.

<120> Elongation Factor P (EFP) And Assays And Antimicrobial Treatments Related To The Same

<130> PUJ-0041

<140> Not Yet Available
<141> Herewith

<150> 60/117,473
<151> 1999-01-27

<160> 3

<210> 1
<211> 185
<212> PRT
<213> *Staphylococcus aureus*

<220>
<223>

<400> 1

Met Ile Ser Val Asn Asp Phe Lys Thr Gly Leu Thr Ile Ser Val Asp
1 5 10 15

Asn Ala Ile Trp Lys Val Ile Asp Phe Gln His Val Lys Pro Gly Lys
20 25 30

Gly Ser Ala Phe Val Arg Ser Lys Leu Arg Asn Leu Arg Thr Gly Ala
35 40 45

Ile Gln Glu Lys Thr Phe Arg Ala Gly Glu Lys Val Glu Pro Ala Met
50 55 60

Ile Glu Asn Arg Arg Met Gln Tyr Leu Tyr Ala Asp Gly Asp Asn His
65 70 75 80

Val Phe Met Asp Asn Glu Ser Phe Glu Gln Thr Glu Leu Ser Ser Asp
85 90 95

Tyr Leu Lys Glu Glu Leu Asn Tyr Leu Lys Glu Gly Met Glu Val Gln
100 105 110

Ile Gln Thr Tyr Glu Gly Glu Thr Ile Gly Val Glu Leu Pro Lys Thr
115 120 125

Val Glu Leu Thr Val Thr Glu Thr Glu Pro Gly Ile Lys Gly Asp Thr
130 135 140

Ala Thr Gly Ala Thr Lys Ser Ala Thr Val Glu Thr Gly Tyr Thr Leu
145 150 155 160

Asn Val Pro Leu Phe Val Asn Glu Gly Asp Val Leu Ile Ile Asn Thr
165 170 175

Gly Asp Gly Ser Tyr Ile Ser Arg Gly
180 185

<210> 2
<211> 188
<212> PRT
<213> *Escherichia coli*

<220>
<223>

<400> 2

Met Ala Thr Tyr Tyr Ser Asn Asp Phe Arg Ala Gly Leu Lys Ile Met
1 5 10 15

Leu Asp Gly Glu Pro Tyr Ala Val Glu Ala Ser Glu Phe Val Lys Pro
20 25 30

Gly Lys Gly Gln Ala Phe Ala Arg Val Lys Leu Arg Arg Leu Leu Thr
35 40 45

Gly Thr Arg Val Glu Lys Thr Phe Lys Ser Thr Asp Ser Ala Glu Gly
50 55 60

Ala Asp Val Val Asp Met Asn Leu Thr Tyr Leu Tyr Asn Asp Gly Glu
65 70 75 80

Phe Trp His Phe Met Asn Asn Glu Thr Phe Glu Gln Leu Ser Ala Asp
85 90 95

Ala Lys Ala Ile Gly Asp Asn Ala Lys Trp Leu Leu Asp Gln Ala Glu
100 105 110

Cys Ile Val Thr Leu Trp Asn Gly Gln Pro Ile Ser Val Thr Pro Pro
115 120 125

Asn Phe Val Glu Leu Glu Ile Val Asp Thr Asp Pro Gly Leu Lys Gly
130 135 140

Asp Thr Ala Gly Thr Gly Gly Lys Pro Ala Thr Leu Ser Thr Gly Ala
145 150 155 160

Val Val Lys Val Pro Leu Phe Val Gln Ile Gly Glu Val Ile Lys Val
165 170 175

Asp Thr Arg Ser Gly Glu Tyr Val Ser Arg Val Lys
180 185

<210> 3
<211> 29
<212> RNA
<213>

<220> Artificial Sequence
<223> Synthetic oligoribonucleotide

<400> 3

gggaauucgg agguuuaaaa auggguaaa 29